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# Comparative Effects of Di(*n*-Butyl) Phthalate Exposure on Fetal Germ Cell Development in the Rat and in Human Fetal Testis Xenografts

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**Running head:** DBP effects on germ cells in human and rat fetal testis.

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## Abstract

**Background: P**hthalate exposure induces germ cell effects in the rat fetal testis. Whilst experimental models have shown that the human fetal testis is insensitive to the steroidogenic effects of phthalates, the effects on germ cells are less explored.

**Objectives:** To identify the effects of phthalate exposure on human fetal germ cells in a dynamic model and to establish if the rat is an appropriate model for investigating such effects.

**Methods:** Immunohistochemistry, immunofluorescence and quantitative real-time PCR for Sertoli and germ cell markers on vehicle/di(*n*-butyl) phthalate (DBP)-exposed rat testes and human fetal testis xenografts. This included analysis of germ cell differentiation markers, proliferation markers and cell adhesion proteins.

**Results:** In both rat and human fetal testes, DBP exposure induced similar germ cell effects, namely germ cell loss (predominantly undifferentiated), induction of multinucleated gonocytes (MNGs) and aggregation of differentiated germ cells, although the latter occurred rarely in the human. The mechanism for germ cell aggregation/MNG induction appears to be loss of Sertoli-germ cell membrane adhesion, probably due to Sertoli cell microfilament redistribution.

**Conclusions:** Our findings provide the first comparison of DBP effects *in vivo* on germ cell number, differentiation and aggregation in human and rat. They show comparable effects on germ cells in both species, but the effects in the human were muted compared with the rat. Nevertheless, phthalate effects on germ cells have potential implications for the next generation, which merits further study. Our results show that the rat is a human-relevant model in which to explore the mechanisms for germ cell effects.

## Introduction

*In utero* exposure of rats to high doses of certain phthalate esters, such as diethylhexyl phthalate (DEHP) or di(*n*-butyl) phthalate (DBP), impairs steroidogenesis by the fetal testis, resulting in postnatal disorders such as hypospadias, cryptorchidism and impaired spermatogenesis (Drake et al. 2009; Johnson et al. 2012; Lehmann et al. 2004; Thompson et al. 2004). In humans, these disorders are thought to comprise a 'testicular dysgenesis syndrome' (TDS) (Skakkebaek et al. 2001), for which the DBP-exposed Wistar rat may be a useful model to dissect the underlying mechanisms (Fisher et al. 2003; Sharpe and Skakkebaek 2008). However, in contrast to the inhibitory effects of DBP exposure on the fetal rat testis, the human fetal testis appears to be insensitive to the steroidogenic effects of DBP, based on studies involving *in vitro* and xenograft models (Heger et al. 2012; Lambrot et al. 2009; Mitchell et al. 2012; Albert and Jégou 2014; Spade et al. 2014).

DEHP/DBP exposure also induces germ cell effects in the fetal rat testis, namely induction of multinucleated gonocytes (MNGs) (Ferrara et al. 2006; Mylchreest et al. 2002; Parks et al. 2000) and aggregation of germ cells in the seminiferous cords (Barlow and Foster 2003; Kleymenova et al. 2005). These changes are only evident from e19.5 to e21.5 in the rat, and are thus confined to differentiated germ cells (i.e. absent OCT3/4 expression) (Ferrara et al. 2006; Jobling et al. 2011). Indirect evidence (Jobling et al. 2011; Kleymenova et al. 2005) suggests that these germ cell changes may be secondary to effects on Sertoli cells. However, DEHP/DBP exposure also induces a reduction in germ cell number that is divorced temporally from aggregation. This effect is confined to the period in the rat when germ cells are undifferentiated (expressing

OCT3/4) and proliferating, namely e13.5-e17.5 (Jobling et al. 2011), and can cause up to 40% reduction in germ cell number by birth (Jobling et al. 2011).

DEHP/MEHP induces germ cell loss and MNGs *in vitro* using human fetal testis explants (Chauvigne et al. 2009; Habert et al. 2009; Lambrot et al. 2009; Lehraiki et al. 2009; Muczynski et al. 2012) and DBP exposure induces MNGs in human fetal testis xenografts (Heger et al. 2012). However, none of these studies identified if the phthalate effects were dependent on the stage of germ cell differentiation, which appears critical *in vivo* in rats.

Our present studies therefore sought to: (1) compare the effects of DBP exposure on germ cell aggregation and MNG induction in the rat and in an established human fetal testis xenograft model (Mitchell et al. 2010, 2012); (2) establish if these changes result from impaired Sertoli-germ cell interaction at the membrane level, (3) establish if DBP-induced germ cell loss occurs in the human fetal testis xenograft model and (4) determine whether effects are restricted to the undifferentiated germ cell population. By answering these questions, our studies identify the potential effects of phthalate exposure on fetal human germ cells, and establish that the rat may be an appropriate model for investigating underlying mechanisms (and their consequences). This is important, considering the disparity in phthalate effects on fetal testis steroidogenesis in the rat and human (see above), and the fact that fetal germ cells ultimately give rise to the next generation. Fetal germ cells are also believed to be the cell of origin for testicular germ cell cancers in young men (Rajpert-De Meyts 2006).

## Materials and Methods

### Ethics statement

We treated animals humanely and with regard for alleviation of suffering, according to the Animal (Scientific Procedures) Act 1986 (http://www.legislation.gov.uk/ukpga/-1986/14/contents) and approval by the UK Home Office. Studies were conducted under Project Licence (PPL 60/4564) following review by the University of Edinburgh Animal Research Ethics Committee. We obtained human fetal testes from women undergoing elective termination of pregnancy, who gave written consent; ethical approval was obtained from the Lothian Research Ethics Committee (LREC - 08/ S1101/1).

### DBP treatment of rats

Wistar rats (Harlan, UK) were housed in ventilated cages (2000P cages, Tecniplast, Italy; 4-6 adult female rats per cage) and had access ad libitum to sterile water and a soy-free breeding diet (RM3(E); SDS, Dundee, Scotland). We carefully controlled housing conditions [lights on at 0700, off at 1900 h, temperature 19–21 C, humidity 45-65%, GOLD shavings and LITASPEN standard bedding (SPPS, Argenteuil, France)]. Animals were housed for a minimum of 2 weeks prior to use in experimental studies. We randomly allocated time-mated females to receive either 0 (control) or 4, 20, 100 or 500mg/kg DBP (Sigma-Aldrich Company Ltd, Dorset, UK) in 1ml/kg corn oil, daily by oral gavage. Treatments were administered between 09:00-10:30h, commencing on embryonic (e) day 13.5 until the day prior to culling (or as indicated otherwise). DBP was 99% pure according to the supplier. All treatments were performed in a single animal facility at the University of Edinburgh. The weight of the female rats prior to the start of treatment was 266.4-319.8 grams and no generalized adverse effects of the exposure to DBP

were observed. There was no significant effect of the treatment on litter size or sex ratio. We sampled male offspring on e17.5, e21.5 or postnatal day (day) 4, time points chosen to reflect the period before, during and after the appearance of DBP-induced MNGs and gonocyte aggregation, respectively. We used 12-14 animals from 3-5 litters per treatment and all experiments reported below used animals from each of these litters. We killed pregnant dams by $CO_2$ inhalation followed by cervical dislocation. Fetuses were removed, decapitated, and placed in ice-cold PBS (Sigma-Aldrich). Day 4 pups were housed with their natural mothers from birth and were killed by cervical dislocation. Fetuses/pups were transported immediately to the laboratory and testes were removed by microdissection. Testes were fixed for 1h in Bouin's fixative (3h for pnd4 testes), then transferred the testes to 70% ethanol. We used representative fetuses for the quantitative and immunohistochemical studies below. For each e21.5 fetus, one testis was fixed as above, whilst the other testis was snap frozen and stored at -70°C for gene expression analysis.

**Human fetal testis xenografts**

Male CD1 nude mice (Charles River, Margate, UK) were used as host mice for human fetal testis xenografts. Mice were housed in individually ventilated cages with free access to sterile food and water. Human fetal testis tissue (14-20 weeks; n = 7) was xenografted subcutaneously into host mice as previously described (Mitchell et al. 2010, 2012). We injected host mice carrying human fetal testes xenografts (5-6 per mouse) subcutaneously every 72h with human chorionic gonadotropin (hCG; 20 IU, three times a week; Pregnyl; Organon Laboratories, Cambridge, UK), in 0.9% (w/v) saline containing 1% (v/v) fetal bovine serum, to mimic the normal *in utero* environment. We then treated host mice with vehicle or 500mg/kg/day DBP for 21 days as

described previously (Mitchell et al. 2012). We fixed xenografts in Bouin's fixative for 1h and transferred these to 70% ethanol and processed them into paraffin blocks using standard procedures.

**Immunohistochemistry**

We used immunohistochemistry for VASA (DDX4/MVH) to identify germ cells in rat testes for analysis of aggregation using image analysis (See 'Measurement of germ cell aggregation in rat testes' below). Similarly, we performed immunohistochemistry for MAGE-A4 (expressed only in differentiated germ cells) and Anti-Müllerian hormone (AMH; Sertoli cells) on human fetal testis xenograft sections. Antibodies and detection reagents are listed in Table 1. For each experiment, we included a negative control (primary antibody replaced with appropriate normal serum). We used protocols described previously (Jobling et al. 2011; McKinnell et al. 2013; Mitchell et al. 2010; van den Driesche et al. 2012).

**Double immunofluorescence for APC/espin, vimentin/N-cadherin and APC/N-cadherin**

To establish if germ cell aggregation in rats resulted from impaired Sertoli-germ cell interaction at the cell membrane, we performed double fluorescence immunohistochemistry for N-cadherin + vimentin, and for APC + espin and analyzed using confocal laser scanning microscopy. Similarly, we co-stained vehicle and DBP-exposed human fetal testis xenograft sections for N-cadherin and APC. Antibodies and detection reagents are listed in Table 1. We used previously described procedures (Jobling et al. 2011; McKinnell et al. 2013).

**Triple immunofluorescence for OCT3/4, MAGE-A4 and Ki67**

To investigate if DBP exposure of human fetal testis xenografts affected germ cell number or proliferation, we undertook triple fluorescence immunohistochemistry for the undifferentiated (pluripotent) germ cell marker OCT3/4, differentiated germ cell marker MAGE-A4 and proliferation marker Ki67, as described elsewhere (Mitchell et al. 2010). Antibodies and reagents are listed in Table 1.

**Measurement of germ cell aggregation in rat testes**

As all germ cells in the perinatal rat testis immunostain intensely for VASA in their cytoplasm, we utilized this to develop an objective method for measuring germ cell aggregation, based on average germ cell cluster size, in testes from control and DBP-exposed rats at e17.5, e21.5 and postnatal day 4. We used stereological methods adapted from previous use to evaluate fetal Leydig cell aggregation (Mahood et al. 2005, 2007) and sections from at least five fetuses from 3-5 litters for analysis. In e21.5 controls, germ cells were in clusters that varied in size from <20-2800 arbitrary units, with most sized <1000 units (see Fig. 1A). In e21.5 DBP-exposed animals, clusters were fewer and, on average, 15% of seminiferous cords contained clusters >3000 units, a size rare in controls (Fig. 1A). A cut-off of >3000 units discriminated best between control and DBP-exposed animals at e21.5, and was therefore used for all aggregation analyses. Germ cell number determination at e21.5 after *in utero* exposure to DBP from e13.5-e15.5 or e19.5-e20.5 also used VASA staining and stereology as described by Jobling et al. (2011).

**Quantification of MNGs in human fetal testis xenografts and fetal rat testes**

AMH is expressed in Sertoli cell cytoplasm but not in germ cells, so we used AMH immunostaining (See 'Immunohistochemistry' above) to facilitate identification of germ cells

and multinucleated gonocytes (MNGs), and to quantify the frequency of MNGs in human fetal testis xenografts and in e21.5 fetal rat testes. We analyzed two sections per xenograft and one complete testis cross-section for fetal rats, using an Axio-Imager microscope (Carl Zeiss Ltd, Cambridge, UK) fitted with a Hitachi HV-C20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). Image-Pro 6.2 software with Stereologer plug-in (MagWorldwide, Wokingham, UK) was used to select random fields within the tissue (15-25 fields per section). All germ cells and MNGs in each field were counted separately using the Stereologer manual tagging function, and the prevalence of MNGs was calculated as a percentage of the total counted (≥350 germ cells + MNGs per section).

**Quantification of germ cell differentiation and proliferation in human fetal testis xenografts**

We generated high resolution, tiled confocal scanning laser microscopy images of complete human fetal testis xenograft sections co-stained for OCT3/4, MAGE-A4 and Ki67 and then quantified germ cell subpopulations and proliferation indices. We counted all germ cells within each section according to their protein expression profile and proliferation status using Zen 2011 software (Carl Zeiss Ltd., Cambridge, UK). For each xenografted testis, we used 2-4 recipient mice for both vehicle and DBP treatments, and germ cell number and differentiation status were determined; we then compiled the average number per treatment and fetus. The total number of germ cells counted ranged from 43-457 in different xenografts.

**Image capture**

We examined and photographed non-fluorescent images using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a Canon DS6031 digital camera (Canon Europe,

Amsterdam, The Netherlands). We captured all fluorescent images using a Zeiss LSM 710 confocal microscope (Carl Zeiss Ltd.). We compiled images using Adobe Photoshop v12 (Adobe, San Jose, CA, USA).

**Gene expression analysis in e21.5 rat fetal testes samples**

For quantitative analysis of gene expression, we extracted and used total RNA from e21.5 testes to prepare random hexamer primed cDNA (van den Driesche et al. 2012). We performed quantitative real time PCR (qRT-PCR) using the ABI Prism Sequence Detection System (Applied Biosystems). We determined the expression of rat *Apc*, *Espn*, *Cdh2* and *Vim* mRNA using the Roche Universal Probe Library (*Apc* forward primer: 5'-CTTCGTGTACGGCAGCTCTT-3', reverse primer: 5'-GCAGTTTCATGCTTGCTCTG-3', probe number 127 Cat no. 04693639001; *Espn* forward primer: 5'-CACCCTCTCCAACTATGACTCC-3', reverse primer 5'-GCTCTGTAAGTCTGAGGATCTGG-3', probe number 25 Cat no. 04686993001; *Cdh2* forward primer: 5'-TGTTCCAGAGGGATCAAAGC-3', reverse primer: 5'-GAGAGGATCCTGTACCTCAGCA-3', probe number 122 Cat no. 04693566001; *Vim* forward primer: 5'-CAGGAAGCTGCTGGAAGG-3', reverse primer: 5'-GGAAGTGACTCCAGGTTAGTTTCT-3', probe number 108 Cat no. 04692276001; Roche Applied Sciences, Burgess Hill, UK). We corrected expression of each gene using a ribosomal 18S internal control (Applied Biosystems Cat no. 4308329). We analyzed all samples in triplicate and made relative comparison to adult testis control cDNA using the ddCt method. We analyzed 12 animals for the control group (from 3 litters) and 14 animals for the DBP group (from 5 litters).

### Statistical analysis

Most of the rat data was derived from 1 animal/litter and was thus analyzed using either Student's *t*-test or one-way ANOVA followed by the Bonferroni post hoc test; where several animals were used per litter (mRNA expression analysis), within-litter effects were accounted for by using two-factor ANOVA. We analyzed human fetal testis xenograft experiments using the paired *t*-test (MNG analysis) or two-way ANOVA (germ cell counts), the latter to take into account of within-fetus effects. We performed analyses using GraphPad Prism 5 (GraphPad Software Inc.).

## Results

### Germ cell aggregation in fetal rat testes and human fetal testis xenografts

DBP-induced rat germ cell aggregation is evident at e21.5 but largely resolves by postnatal day 4 (Fig. 2A-F), findings we confirmed quantitatively (Fig. 1B). Unexpectedly, e17.5 germ cells appeared to be aggregated in both control and DBP-exposed animals (Fig. 2A,D). However, whereas these germ cell aggregates underwent 'disaggregation' in controls between e17.5 and e21.5, this process failed to occur by e21.5 in DBP-exposed animals (Fig. 2E). We evaluated the dose-dependence of DBP-induced germ cell aggregation at e21.5, and showed that aggregation was induced by exposure to DBP at all doses of 20 mg/kg and higher (Fig. 1B).

Using xenograft sections immunostained for MAGE-A4 (Figs. 2G-I) or for OCT3/4 (not shown) we found germ cell aggregation in only 4 out of 54 DBP-exposed xenografts from 7 fetuses and these 4 xenografts were all derived from a single fetus (Fig. 2H). The remaining 50 DBP-exposed xenografts were indistinguishable from vehicle-exposed xenografts (Figs. 2G,I). We confirmed this by immunostaining for the Sertoli cell marker AMH (Fig. 2J-L). We concluded

that DBP-exposure only results sporadically in germ cell aggregation in xenografted human testes but, when aggregation is induced, it affects differentiated (MAGE-A4$^{pos}$) germ cells, as in rats.

**Sertoli-germ cell interaction**

To study the role of Sertoli-germ cell interactions in DBP-induced germ cell aggregation, we examined expression of cell-cell adhesion molecules, APC, espin, vimentin and N-cadherin (Bartles et al. 1996; Chen et al. 1999; Ivaska et al. 2007; Kleymenova et al. 2005; Strelkov et al. 2003; Tanwar et al. 2011; Vogl et al. 2008). In control rats at e17.5 and e21.5, espin was expressed in distal Sertoli cell cytoplasm that extended into 'fingers' that encircled each germ cell (Fig. 3A,B). APC expression was restricted to germ cells but co-localized with espin (expressed in Sertoli cells) where Sertoli and germ cells were apposed, visible as yellow staining in Fig. 3A and B (inserts). This is interpreted as evidence for Sertoli-germ cell interaction at the membrane level, and was evident at all ages in controls. In contrast, in DBP-exposed rats at e17.5 and e21.5, Sertoli cell cytoplasm was not evident between the germ cells and there was no overlapping of espin and APC expression (Fig. 3C,D), indicative of absence of direct Sertoli-germ cell membrane contact, coincident with germ cell aggregation. However, by day 4 Sertoli cell espin expression in testes of DBP-exposed rats was largely restored to normal with cytoplasmic 'fingers' extending around individual germ cells and interacting with APC at the membrane level, as in controls (data not shown). Immunostaining for Sertoli cell filaments vimentin and N-cadherin showed the former was confined to basal, and the latter to apical, Sertoli cell cytoplasm, but both showed evidence for retraction in DBP-exposed rats, similar to that found for espin (Fig. 3E-H). As mRNA expression for *Apc*, *Espn*, *Cdh2* and *Vim* was

unchanged at e21.5 after DBP exposure (Fig. 4), we presume that the DBP-induced microfilament changes results from protein redistribution, although DBP-induced protein degradation and/or translational regulation cannot be excluded.

Immunostaining for APC and N-cadherin in control human fetal testis xenografts demonstrated Sertoli cell cytoplasm distribution around the germ cells (Fig. 3I,J), similar to that in rats. However in the few seminiferous cords that exhibited germ cell aggregation after exposure to DBP there was a lack of Sertoli cell cytoplasmic 'fingers' extending around the germ cells, again very comparable to the findings in rats.

**MNGs in e21.5 rat testes and human fetal testis xenografts after exposure to DBP**

We used AMH stained sections, in which germ cells remained unstained, to determine the appearance of MNGs in fetal rat testes and human fetal testis xenografts after DBP exposure. In e21.5 testes of DBP-exposed rats, we observed a significant increase in MNGs (expressed as a percentage of all germ cells) compared to the vehicle-exposed group (Controls 0%; DBP 3.9%, $p = 0.0016$; Fig. 5A-C). In human fetal testis xenografts, we observed a low frequency (0.8%) of MNGs in vehicle-exposed controls, similar to that described by others (Heger et al. 2012). We also observed a significant increase in incidence (1.76%) of MNGs in xenografts from DBP-exposed compared to vehicle-exposed animals ($p=0.0034$; Fig. 5D-F).

**DBP effects on germ cell proliferation and number in human fetal testis xenografts and on germ cell number in fetal rat testes**

We triple immunostained xenografts for OCT3/4 (undifferentiated germ cell marker), MAGE-A4 (differentiated germ cell marker) and the proliferation marker Ki67 (Fig. 6A-D). For vehicle- and DBP-exposed xenografts, the majority of undifferentiated germ cells were immunopositive for

Ki67 indicating active proliferation, whereas Ki67$^{pos}$/MAGE-A4$^{pos}$ germ cells were rare (Fig. 6E), indicating that germ cells stop proliferating after differentiation.

In rats only exposure to DBP from e13.5-e15.5 (when germ cells express OCT3/4 and proliferate) reduced the number of germ cells at e21.5, whereas DBP exposure from e19.5-e20.5 (when germ cells have differentiated and stopped proliferating) had no significant effect (Fig. 7A). In human fetal testis xenografts we observed a small, but significant reduction in total number of germ cells per xenograft as a result of DBP exposure (Fig. 7B). Similarly to the fetal rat, in human fetal testis xenografts, DBP-exposure caused a significant reduction ($p = 0.041$) in number of OCT3/4$^{pos}$ germ cells in comparison to vehicle-exposed controls (Fig. 7C), whereas there was a non-significant reduction in the number of MAGE-A4$^{pos}$ germ cells ($p = 0.057$; Fig. 7D). Thus DBP exposure results in germ cell loss in the human testis during the developmental stage examined (14-20 weeks), with particular loss of undifferentiated OCT3/4$^{pos}$ cells. In the rat a similar period of sensitivity was found to DBP-exposure (i.e. e13.5-e15.5) (Fig. 7A).

## Discussion

Concerns about the potential human health effects of phthalates such as DEHP and DBP have been tempered with the demonstration that, unlike in the rat, the human fetal testis appears insensitive to the anti-steroidogenic effects of DBP in a range of model systems (Heger et al. 2012; Lambrot et al. 2009; Mitchell et al. 2012; Albert and Jégou 2014; Spade et al. 2014). In contrast, the available evidence suggests that these same phthalates may adversely affect fetal germ cells in both rodents (Ferrara et al. 2006; Jobling et al. 2011) and humans (Heger et al. 2012; Lehraiki et al. 2009; Muczynski et al. 2012), although the underlying mechanisms are unexplored. Our present studies sought to address these deficiencies by comparing the effects of

DBP exposure *in vivo* on germ cells in the fetal rat testis with its effects on germ cells in the human fetal testis in an established xenograft model. Our findings identify periods of germ cell sensitivity to the effects of DBP exposure in the rat (e13.5-e15.5) and human fetal testis (14-20 weeks of gestation). In rats this coincides with the period when germ cells are undifferentiated (OCT3/4$^{pos}$). In humans, differentiation of germ cells from OCT3/4$^{pos}$ to MAGE-A4$^{pos}$ occurs asynchronously over an extended period from first trimester to early postnatal life resulting in a heterogeneous population of germ cells at any given age during fetal development (Mitchell et al, 2008). The predeliction for DBP-induced loss of OCT3/4$^{pos}$ germ cells may indicate a sensitive stage of differentiation rather than gestational age and this requires further clarification; specifically there may be more marked effects in earlier gestations when more of the germ cells are undifferentiated. In contrast, the development of germ cell aggregation and/or MNG was confined to differentiated germ cells in both fetal rats and humans, although aggregation appears to be a rare occurrence in the human when compared with the rat. We also show that the underlying mechanism for DBP-induced germ cell aggregation is probably a loss of Sertoli-germ cell membrane interaction, potentially due to microfilament redistribution within Sertoli cells. These findings provide new insights into the potential human testicular effects of fetal phthalate exposure.

The key present finding is that exposure of mice bearing human fetal testis xenografts to a high dose of DBP results in a small loss of germ cells, affecting undifferentiated (OCT3/4 expressing) fetal germ cells in particular. As it is the undifferentiated, rather than the differentiated, germ cells that are highly proliferative (this study and Mitchell et al. 2010, 2014), and as undifferentiated germ cells are present throughout human gestation (Mitchell et al. 2010), it is

possible that protracted fetal DBP exposure may cause progressive germ cell depletion in the human fetal testis. Similarly, MEHP exposure *in vitro* causes apoptotic germ cell loss in human fetal testis explants (Lambrot et al. 2009; Muczynski et al. 2012). Our xenograft studies have not investigated the dose-dependence of the germ cell changes induced by DBP, because of the limited supply of human tissue, but it remains an important issue in terms of risk evaluation for the human fetus. In this regard, it is emphasized that the present studies used a dose of DBP (500mg/kg/day) which is ~30,000 times higher than the reported exposure levels for women from the general population (Heudorf et al. 2007). It is also possible that undifferentiated germ cells that survive DBP exposure might be affected in other ways; notably, undifferentiated fetal germ cells are thought to give rise to carcinoma-in-situ (CIS) cells in humans, from which testicular germ cell tumors develop in adulthood (Rajpert-De Meyts 2006). As it is the OCT3/4$^{pos}$/MAGE-A4$^{neg}$ population of undifferentiated germ cells that has subsequent invasive potential (Mitchell et al. 2014), this is an important avenue for further study. Overall, our findings show that DBP effects on fetal germ cells in the human are less pronounced than in the rat at the same dose, but the similarity of the types of effect observed with those in the rat suggest that the latter may represent a suitable model for evaluating mechanisms of DBP-induced germ cell effects relevant to the human.

In the fetal rat, induction of MNGs (Fisher et al. 2003; Kleymenova et al. 2005; Mylchreest et al. 2002) and abnormal germ cell aggregation are also consequences of DBP exposure and, as the present and previous (Ferrara et al. 2006; Jobling et al. 2011) data show, these effects are confined to differentiated germ cells. We postulate that the formation of MNGs may reflect a failure to maintain cytoplasmic intercellular bridges. Moreover, germ cell aggregation was

induced dose-dependently by DBP, even with relatively low doses (20 mg/kg/day). In human fetal testis xenografts, DBP-induction of germ cell aggregation was an infrequent occurrence in our studies, but, as in the rat, was confined to differentiated (MAGE-A4$^{pos}$) germ cells. Of the 7 fetal testes exposed to DBP after xenografting, germ cell aggregates were only found in 4 of 54 xenografts, all of which were derived from a single 19-week fetus. It may be that, because undifferentiated and differentiated germ cells are intermixed in human and there is no defined 'early' or 'late' period in terms of germ cell differentiation, that induction of aggregation in the human is simply less possible, as opposed to the rodent fetal testes where germ cell differentiation occurs synchronously (McKinnell et al. 2013; Mitchell et al. 2010). As DBP-induction of MNGs in rodents coincides with induction of germ cell aggregation, it may be that the underlying cause is the same. In this respect, the present and previous studies using human fetal testis xenografts (Heger et al. 2012) or *in vitro* cultures (Lehraiki et al. 2009) have also shown phthalate induction of MNGs.

The present findings extend previous observations by providing more direct evidence that DBP-induced germ cell aggregation results from dissolution of Sertoli-germ cell membrane cross-linking (via espin-APC) which in turn may result from the redistribution of microfilaments, such as vimentin and N-cadherin. APC, espin, N-cadherin and vimentin have established roles in Sertoli-germ cell interactions (Bartles et al. 1996; Chen et al. 1999; Kleymenova et al. 2005; Strelkov et al. 2003; Tanwar et al. 2011; Vogl et al. 2008). Expression of APC and espin was co-localized at the Sertoli-germ cell borders in e17.5 and e21.5 control testes, whereas in DBP-exposed testes this co-localization was absent. Furthermore, immunostaining for Sertoli cell filament markers N-cadherin and vimentin showed a collapse of the filament structures, with loss

of Sertoli cell cytoplasmic 'fingers' extending towards the centre of the seminiferous cords in DBP-exposed rats at all ages. This altered pattern of vimentin staining following DBP exposure in e21.5 fetal rat testes is similar to that previously described (Kleymenova et al. 2005). Interestingly, the DBP-exposed human fetal testis xenografts that exhibited germ cell aggregation also showed an altered staining pattern for N-cadherin and APC similar to the rat, with a lack of Sertoli cell cytoplasmic 'fingers' extending around the germ cells and loss of N-cadherin-APC interaction at the membrane level (espin could not be studied in the human). The present findings therefore provide a basis for further studies to investigate the mechanism of DBP-induced germ cell aggregation.

## Conclusions

Our results show that DBP exposure of human fetal testis xenografts results in similar, although more muted, germ cell effects as seen in the rat, namely germ cell loss during fetal life with a predominant loss of undifferentiated (OCT3/4$^{pos}$) germ cells, induction of MNGs and (infrequent) germ cell aggregation, the latter two effects being confined to differentiated germ cells in both species. Moreover, our results suggest similar DBP-induced loss of Sertoli-germ cell membrane contact amongst differentiated germ cells. In view of these overall similarities, we suggest that the rat may be an appropriate model in which to study the mechanisms underlying DBP effects on germ cells. Whether fetal germ cell effects would occur in human fetal testes at population-relevant DBP exposure levels remains to be explored.

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**Table 1.** Antibodies used for immunohistochemistry and immunofluorescence, their source, dilution and visualization.

| Antibody | Source | Dilution | Secondary antibody | Visualization |
|---|---|---|---|---|
| Melanoma-associated antigen 4 (MAGE-A4; IHC) | Gift[1] | 1:20 | [a]GAM-b | [b]DAB |
| VASA (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4)/mouse vasa homologue (MVH)) | Abcam | 1:80 | [c]GAR-b | [b]DAB |
| Anti-Müllerian hormone (AMH) | Santa Cruz Biotechnology | 1:500 | [d]RAG-b | [b]DAB |
| Adenomatous polyposis coli (APC) | Fisher Scientific | 1:750 | [e]GAR-p | [f]Tyr-fl |
| Espin | BD Transduction Labs | 1:4000 | [g]GAM-p | [h]Tyr-Cy3 |
| N-cadherin | Zymed Laboratories | 1:4000 | [g]GAM-p | [f]Tyr-fl |
| vimentin | DAKO | 1:750 | [g]GAM-p | [h]Tyr-Cy3 |
| MAGE-A4 (IF) | Gift[1] | 1:100 | [i]ChAM-p | [j]Tyr-Cy5 |
| Octamer-binding transcription factor 3/4 (OCT3/4) | Santa Cruz Biotechnology | 1:100 | [k]ChAG-p | [h]Tyr-Cy3 |
| Ki67 | DAKO | 1:200 | [i]ChAM-p | [f]Tyr-fl |

[1]Dr Giulio Spagnoli, University Hospital, Basel, Switzerland; [a]GAM-b, goat anti-mouse biotin (DAKO Corp., UK); [b]DAB, 3,3'-diaminobenzidine tetrahydrochloride (Vector Labs, UK); [c]GAR-b, goat anti-rabbit biotin (DAKO Corp., UK); [d]RAG-b, rabbit anti-goat biotin (DAKO Corp., UK); [e]GAR-p, goat anti-rabbit peroxidase (DAKO Corp., UK); [f]Tyr-fl, Tyramide Fluorescein (Perkin Elmer, MA, USA); [g]GAM-p, goat anti-mouse peroxidase (DAKO Corp., UK); [h]Tyr-Cy3, Tyramide Cy3 (Perkin Elmer, MA, USA); [i]ChAM-p, chicken anti-mouse peroxidase (DAKO Corp., UK); [j]Tyr-Cy5, Tyramide Cy5 (Perkin Elmer, MA, USA); [k]ChAG-p, chicken anti-goat peroxidase (DAKO Corp., UK); IHC, immunohistochemistry; IF, immunofluorescence.

## Figure legends

**Figure 1.** Analysis of the number and size of germ cell aggregates for a representative control (left) and DBP-exposed rat (right) at e21.5 (A). The dashed line indicates clusters >3000 units as an arbitrary cut-off point. Note the different scales used for vehicle and DBP. The lower panel (B) shows the dose-dependence of DBP-induced germ cell aggregation at e21.5. Note that, unless otherwise indicated, treatment was daily from e13.5-16.5 (e17.5), e13.5-20.5 (e21.5) or from e13.5-21.5 (pnd4). Data was analyzed by one-way ANOVA followed by Bonferroni post hoc test; **$p<0.01$, ***$p<0.001$, in comparison with respective control value. Values are Means ± SEM for 5 animals per group from 5 litters.

**Figure 2.** Germ cell distribution and DBP-induced germ cell aggregation in the rat at embryonic day (e) 17.5, e21.5 and postnatal day (pnd) 4 after treatment with DBP or vehicle (control) and in human fetal testis xenografts after DBP exposure. The upper panels (A-F) illustrate the distribution of germ cells, visualized by immunostaining for VASA (DDX4/MVH). Germ cell aggregation is seen in rat testes of e21.5 DBP-exposed animals (E), whereas testes from vehicle-exposed controls had normal germ cell distribution at e21.5 (B). The lower panels (G-L) show consecutive sections of human fetal testis xenografts from vehicle- and DBP-exposed hosts immunostained for MAGE-A4 (G-I) or AMH (J-L). Note that germ cell aggregations were found in only 4 of 54 DBP-exposed xenografts, all from 1 fetus. Asterisks indicate germ cell aggregation. Scale bar denotes 50μm (A-F) and 100μm (G-L).

**Figure 3.** Effect of exposure to vehicle or DBP (500 mg/kg/day) on Sertoli-germ cell interactions at e17.5 and e21.5 in fetal rat testes and in human fetal testis xenografts. In rats, representative photomicrographs show immunoexpression of APC (green) in germ cells and espin (red) in Sertoli cells (A-D), and (E-H) vimentin (red) and N-cadherin (green) in Sertoli cells on e17.5 and e21.5. In human fetal testis xenografts, APC (red) and N-cadherin (green) immunoexpression were evaluated in xenografts from vehicle- (I) and DBP-exposed hosts (J). Inserts show higher magnification of seminiferous cords. In rats, yellow staining (APC and espin co-localization; white arrows) was absent in DBP-exposed samples at all ages (C-D), compared with vehicle-exposed controls (A-B). Similarly, vimentin and N-cadherin co-staining demonstrated a lack of apical Sertoli cell cytoplasmic extensions after DBP exposure (yellow

arrows) in rats, indicative of withdrawal of Sertoli cell cytoplasm from around and between the germ cells (E-H). Similar loss of Sertoli-germ cell membrane interaction was observed in the limited human fetal testis xenografts that exhibited germ cell aggregation after exposure to DBP (J). White asterisks indicate normal germ cells, yellow asterisks indicate abnormal germ cell aggregation. Note that in rats, treatment was daily from e13.5 until the day before sampling (e17.5, e21.5). Scale bar denotes 50μm.

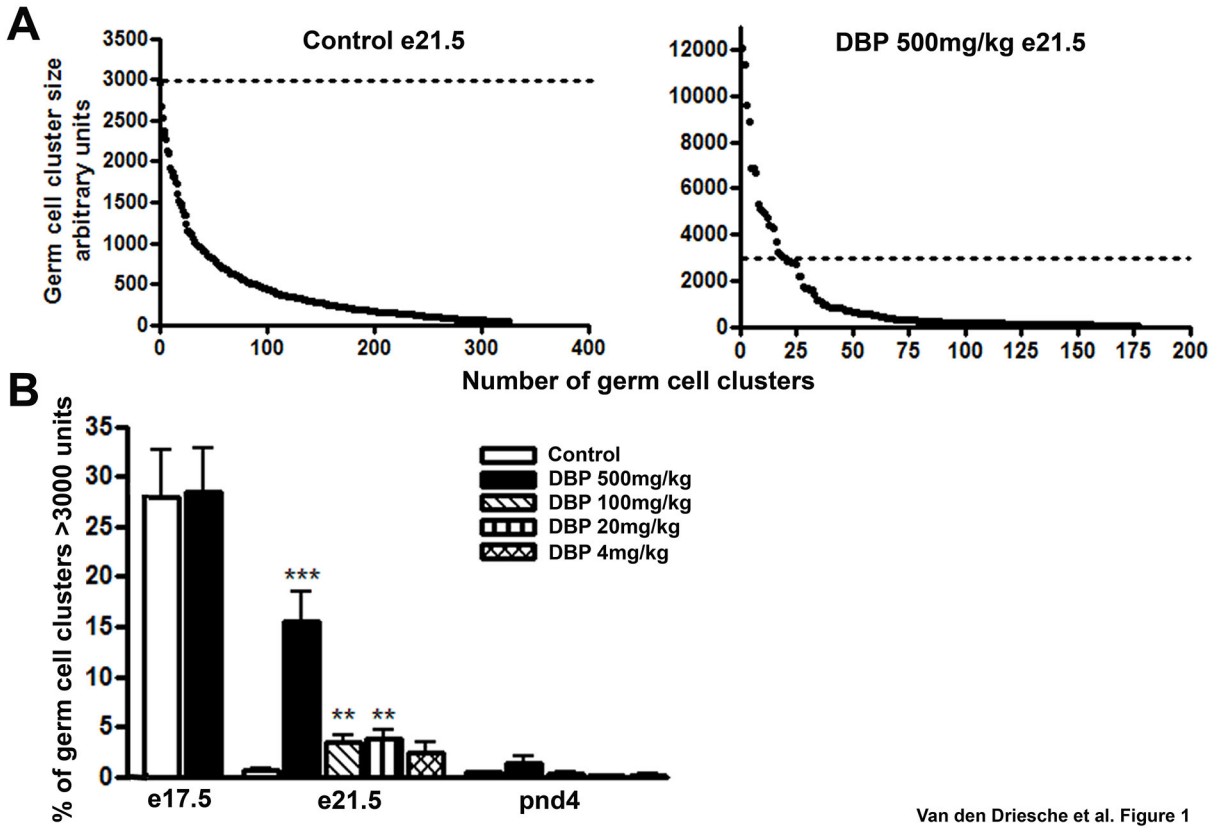
**Figure 4.** Effect of *in utero* exposure of rats to vehicle or DBP (500 mg/kg/day) on the expression of *Apc* (A), *Espn* (B), *Cdh2* (C) and *Vim* (D) in testes at e21.5 using quantitative real-time RT-PCR. Values are Means ± SEM for 12 animals for the control group (from 3 litters) and 14 animals for the DBP group (from 5 litters).

**Figure 5.** Effect of exposure to vehicle or DBP (500 mg/kg/day) on the induction of multinucleated gonocytes (MNGs) in e21.5 rat testes (A-C) and human fetal testis xenografts (D-F). AMH immunostaining (brown) highlights an increased number of MNGs (arrows) in both rat (B) and human (E) fetal testis samples after DBP exposure, which was confirmed by quantification (C,F). Two sections were quantified from 1-5 grafts from 7 different fetuses. ** $P<0.01$, in comparison with respective vehicle group. Scale bar denotes 100μm.

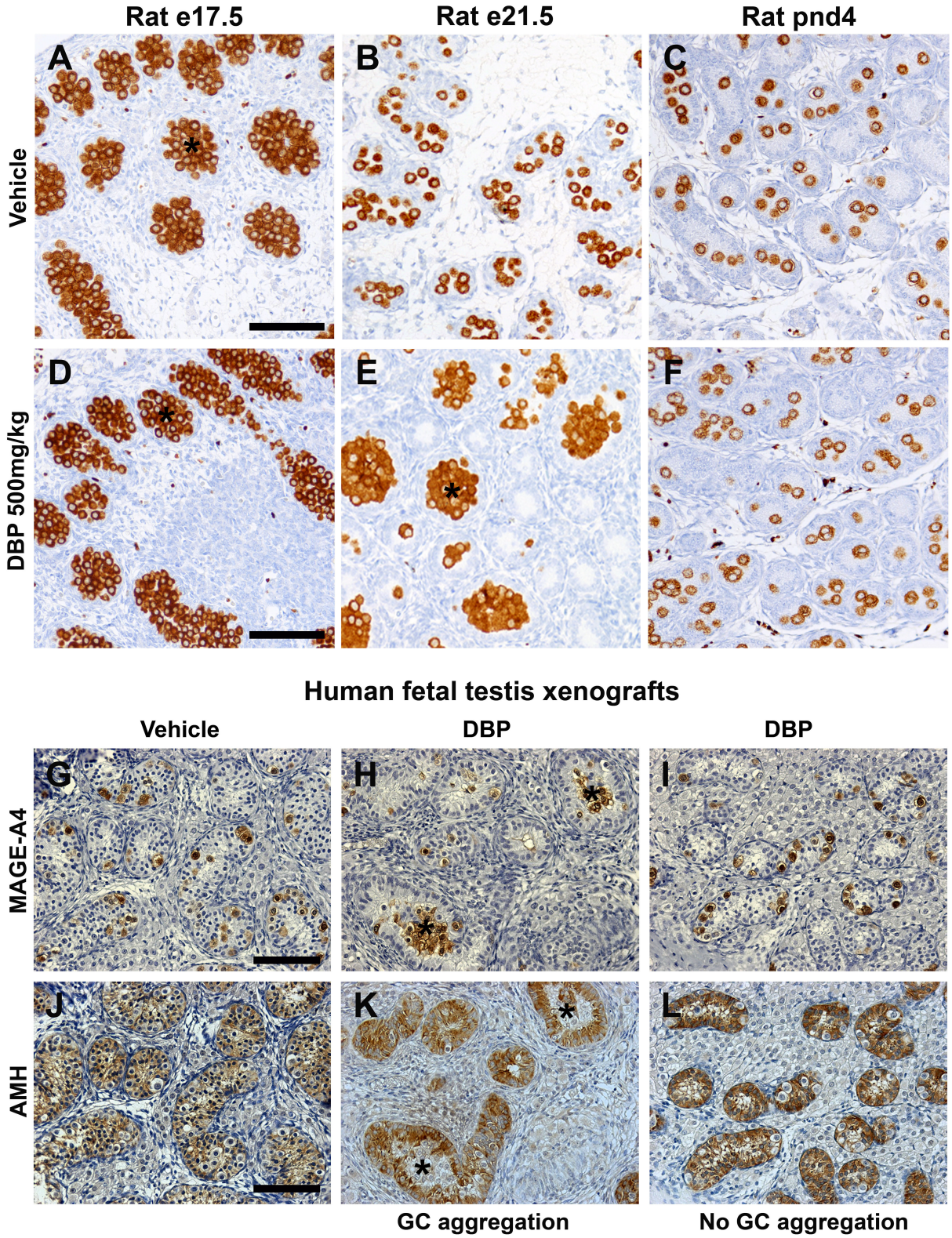
**Figure 6.** Effects of exposure to vehicle (A,C) or DBP (500 mg/kg/day; B,D) on the proliferation (Ki67, green) of undifferentiated (OCT3/4; red) and differentiated (MAGE-A4; blue) germ cell subpopulations in human fetal testis xenografts. Examples of proliferating germ cells expressing OCT3/4 are indicated by arrowheads; differentiated germ cells positive for MAGE-A4, but negative for OCT3/4 and Ki67 immunostaining, are indicated by arrows. (E) The percentage of $OCT3/4^{pos}$ or $MAGE\text{-}A4^{pos}$ germ cells expressing Ki67. No significant differences were found after exposure to DBP. Values are Means ± SEM for n=7 fetuses. Scale bars denote 200μm (A-B) and 50μm (C-D).

**Figure 7.** Effect of early (e13.5-e15.5) or late (e19.5-e20.5) fetal DBP exposure in rats on germ cell number per testis at e21.5 (A), and effects of exposure to vehicle or DBP (500 mg/kg/day) on the total number of germ cells (B), and the numbers of $OCT3/4^{pos}$ (C) and $MAGE\text{-}A4^{pos}$ (D) germ cells in human fetal testis xenografts. Panels B, C and D show mean ± SEM germ cell

numbers for host mice xenografted with testes from individual fetuses (n = 7) and exposed to vehicle or DBP. Rat data was analyzed by unpaired *t*-test (** $P<0.01$, ns = not significant, in comparison with respective vehicle group). Xenograft data were analyzed using two-way ANOVA.




Van den Driesche et al. Figure 1

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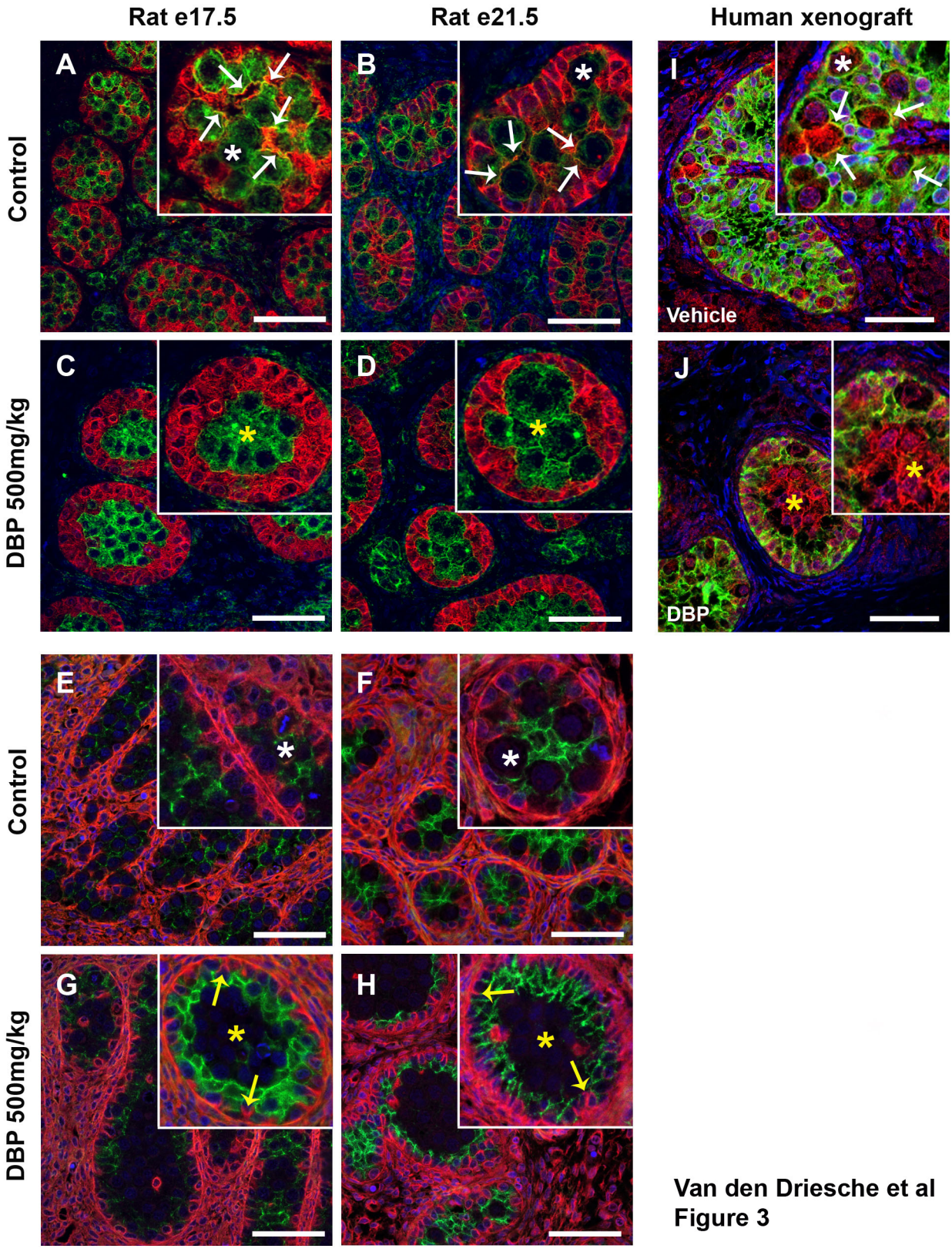
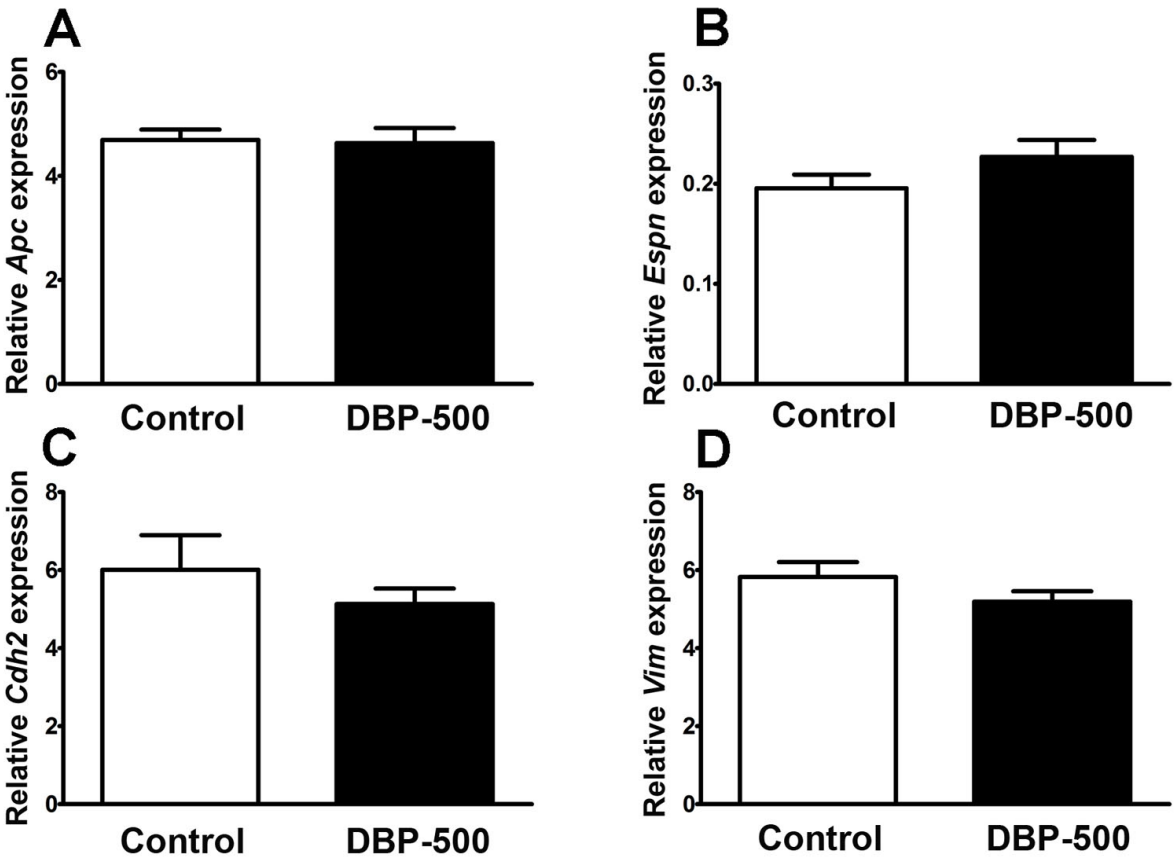



Van den Driesche et al
Figure 3

Van den Driesche et al. Figure 4



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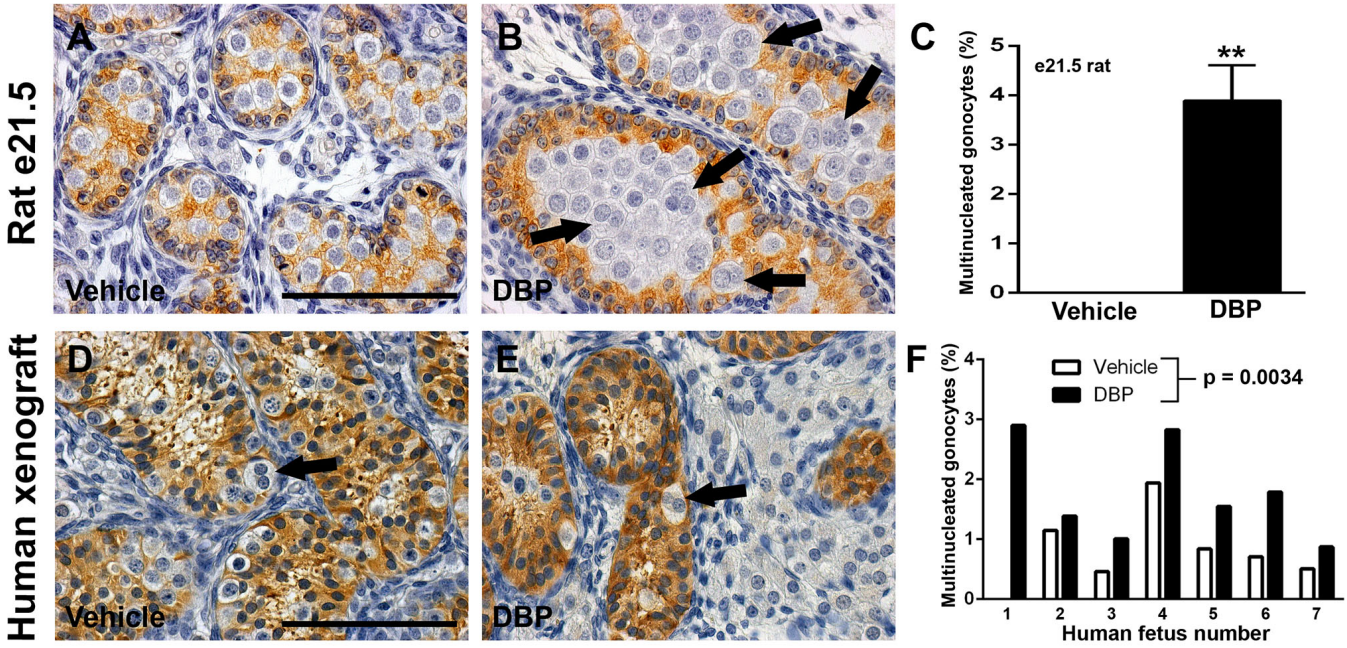
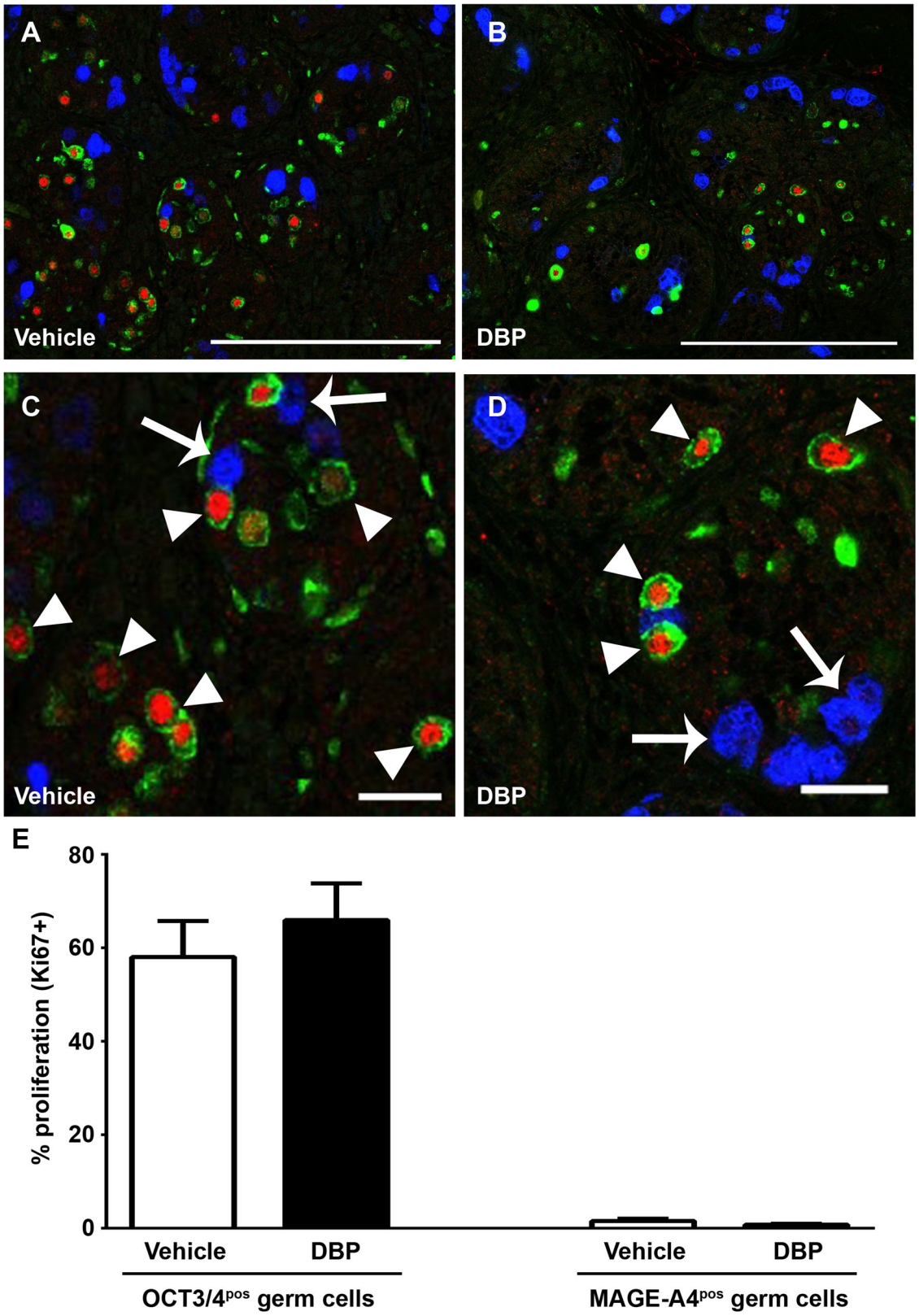


Van den Driesche et al. Figure 6



Van den Driesche et al. Figure 7